REMARKS/ARGUMENTS

First, Applicants would like to thank Examiners Long Le and Jacob Cheu for the courtesies extended during the telephonic interview conducted on October 12, 2005.

In response to the Office Action of September 08, 2005, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Claim Status/Support for Amendments

Claims 1, 39-46 are currently pending. Claims 39 and 42 have been amended herein. Claims 2-38 were cancelled in a previous response. Claim 39-46 remain pending. As discussed during the aforementioned telephonic interview of October 12, 2005, examined claim 1 (Group I) has been deemed to be allowable by the Examiner. Claims 39-46 are drawn to the non-elected invention. Applicants respectfully request rejoinder of the remaining claims (39-46), in accordance with the decision in *In re Ochiai*, since the remaining claims (39-46) are limited to the use of the biopolymer markers of claim 1 (the examined claim of the elected Group I invention). If the biopolymer marker peptide of claim 1 is found to be novel, methods and kits limited to its use should also be found novel.

No new matter has been added by the amendments to the claims made herein.

Claim 39 has been amended in response to suggestions made by the Examiner during the telephonic interview of October 12, 2005. For the sake of clarity "in a manner effective to maximize elucidation of discernible" in claim 39, step (b) has been replaced with --to elucidate--. Support for this amendment can be found throughout the specification as originally filed, see, for example page 35, lines 19-22.

Claim 42 has been amended to define the acronyms for the recited mass spectrometry procedures. These acronyms are well known to those of skill in the art and are defined in various parts of the specification as originally filed, see, for example page 10, lines 2-11.

Drawings

Applicants have provided the Examiner by way of FEDEX express, mailed on October 19, 2005, a Declaration under 37 CFR § 1.132 with attached Figure (entitled "HiQ 1 - (Elusion) Insulin Resistance vs. Normal"), which represents replacement Figure 1. The Examiner asserts in the rejection of claim 1 under 35 USC 112 (first paragraph), discussed below, the results of the photograph in original Figure 1 are "vague and fuzzy" with only one band appearing on one of the insulin resistance patients. As discussed in the aforementioned Declaration, replacement Figure 1 is provided

to more clearly show Band #3 corresponding to betaine/GABA transport protein fragment (SEQ ID NO:2); the biopolymer marker as currently claimed is clearly present in the different insulin resistance patients (lanes 3 and 4; as read from the left). Replacement Figure 1 is merely a duplicate of the original gel made at the time the experiments disclosed in the instant specification were first carried out, thus, replacement Figure 1 presents no new matter into the specification as filed.

Rejection under 35 USC 112, first paragraph

Claim 1, as filed on July 29, 2005, stands rejected under 35 USC 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Examiner asserts that the claim contains subject matter which was not described in the specification in a such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The Examiner now asserts that the instant invention would not enable one of ordinary skill in the art to use this invention without undue experimentation. The Examiner posits that in view of the mass spectral profile of the peptides as in Figures 2 and 3, there is no explanation or illustration of the significance or relationship between the peptide fragments, i.e., SEQ ID NO:2, and

insulin resistance.

The Examiner further asserts the current data in Figure 1 does not demonstrate "up-regulation" phenomena in the insulin resistance patients. The Examiner points to Figure 1 containing the two samples from insulin resistance patients (second and third lane from the left [sic]). The results of the gel electrophoresis are allegedly vague and fuzzy and show only one band vaguely appearing on one of the insulin resistance patients (see lane two of Figure 1). Thus, the Examiner concludes there is no consistency among the insulin resistance patients if the appearance of a fragment peptide (up-regulation) is significant and essential as a biomarker for this specific disease.

Applicants respectfully disagree with the Examiner's interpretation of the claimed invention and submit that the Examiner's assertion regarding lane 2 is incorrect and may have caused the Examiner to misinterpret the results shown in the gel electrophoresis of original Figure 1. Lane 1 is reserved for low molecular weight standards. Lane 2 (as read from the left) corresponds to a diabetes type I patient, not an insulin resistance patient as stated by the Examiner in the instant Office action (see original and replacement Figure 1). Lanes 3 and 4 contain samples obtained from different insulin resistance patients. Lanes 5 and 6 contain sample from Type II diabetes patients. Lanes 7 thru 9

correspond to sample obtained from normal control patients and lane 10 is reserved for high molecular weight standards, as disclosed along the perimeters of the photograph in original Figure 1 and replacement Figure 1.

Band #3 corresponds to betaine/GABA transport protein. The identified betaine/GABA transport protein fragment weighs about 1211.5591 daltons and corresponds to the biopolymer marker that is currently claimed (SEQ ID NO:2), see page 46, lines 4-10. Band #3 appears in both insulin resistance patients, that is, lanes 3 and 4, respectively (see replacement Figure 1). Band #3 was resolved from the gel that is pictured in Figure 1. Thus, contrary to the Examiner's assertion, replacement Figure 1 does demonstrate "up regulation" phenomena in the insulin resistance patients.

In the interest of furthering prosecution, Applicants filed a Declaration under 37 CFR § 1.132 (October 19, 2005) with attached Figure entitled "HiQ 1 -(Elusion) Insulin Resistance vs. Normal" which now clearly shows a comparison of the protein content of samples obtained from patients having a history of insulin resistance or Type I diabetes with the protein content of samples obtained from patients determined to be normal with regard to insulin resistance and diabetes. Band #3 is clearly decipherable in lanes 3 and 4 (as read from the left) of replacement Figure 1,

as resolved from a sample obtained from a patient with a history of insulin resistance and can be identified as differentially expressed between a disease state (insulin resistance) and a non-disease state (normal). Subsequently Band #3 was excised from the gel and subjected to mass spectrometry (TOF MS/MS; Figures 2 and 3). The resulting mass spectral profile of the currently claimed SEQ ID NO: 2 (Figure 2) was then compared with a database of the sequences of known peptides and the profile identified as a fragment of the betaine/GABA transport protein.

Considering that the betaine/GABA transport protein fragment (SEQ ID NO:2) was identified by differential expression between insulin resistance and normal, it is indicated as a potential disease marker for insulin resistance. The mass spectral profile of SEQ ID NO:2 as shown in Figure 2, as established by the instant invention, can be used as a reference for comparison with test samples. Accordingly, the presence of the mass spectral profile of SEQ ID NO:2 in a sample can potentially identify insulin resistance in the patient from which the sample was obtained, i.e. Figure 2 represents insulin resistance patients.

Thus, contrary to the Examiner's assertions, the instant specification does explain and illustrate the relationship between the claimed peptide fragments and insulin resistance.

The "test of enablement" is whether one reasonably skilled in

the art could make or use the invention from the disclosures in the patent coupled with information known in the prior art without undue experimentation (see MPEP 2164.01) and/or a statement of utility in the specification contains within it a connotation of how to use, 35 USC 112, is satisfied (see MPEP 2164.01(c)).

Furthermore, the decision in *In re Brandstadter* (179 USPQ 286; MPEP 2164.05) has established that the evidence provided by applicant (to overcome an enablement rejection) need not be conclusive but merely convincing to one of skill in the art.

The Examiner appears to provide as evidence of lack of enablement in the instant specification a recent article (Zhang et al., Neurobiology of Aging, Vol. 26, page 207 (2005)), wherein the Examiner believes the authors conducted studies using similar methods as described in the instant invention, i.e., proteomic approaches for two-dimensional gel differential electrophoresis coupled with mass spectrometry analysis. The study by Zhang et al., was aiming to identify biomarkers of common age-related neurodegenerative disease. The authors identified around 30 proteins with >20% change in concentration between older and younger individuals. According to the Examiner, Zhang et al., do not conclude that these proteins as biomarkers, rather, Zhang et al., suggest the data of those proteins are a "value platform" and invite further experimentation and confirmation (see page 214,

right column, second paragraph; left column, last paragraph).

Applicants respectfully disagree with the Examiner's reliance on the article by Zhang et al.

As noted by the Examiner, Zhang et al., was published in 2005 (received October 2003), which is more than 3 years after the filing date of the instant invention (November 21, 2001), thus, Applicants believe the reference is not relevant to the state of the art existing at the filing date of the application and cannot be used to determine whether the instant disclosure is enabled as of the filing date. It has been established that, in general, the Examiner *should* not use post-filing date references to demonstrate the patent is non-enabling (see MPEP 2164.05(a)).

Assuming, *in arguendo*, Zhang et al., is a valid reference the mere fact that something has not been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it (see MPEP 2164.02).

Applicants assert that upon closer inspection the study of Zhang et al., do not parallel the methods disclosed in the instant invention. Zhang et al., used a "shotgun" proteomic approach coupled with liquid chromatography followed by mass spectrometry to identify proteins in human CSF (see abstract; page 208, left column, last paragraph). Specifically, the method of Zhang et al. used pooled CSF samples from 22 younger and 16 older subjects to

generate two pooled samples for proteomic analysis (page 208, right column, penultimate paragraph). Zhang et al., acknowledges the problem with the use of pooled CSF samples is that they were unable to determine if the differences found were due to age-related changes in a single individual, only few individuals or distributed over all participants (page 214, last paragraph).

The claimed methodology of the present invention does not use pooled samples, rather, a sample from an individual patient is obtained and at least one biopolymer marker sequence is isolated from the sample and compared to the biopolymer marker sequence as disclosed in the present invention. Unlike Zhang et al., the presence of the mass spectral profile of SEQ ID NO:2 of the instant invention in a sample can potentially identify insulin resistance in the patient from which the sample was obtained, see for example, page 46, line 17 to page 47, line 5.

The Zhang et al., publication states the aging *markers* (30 identified proteins) need to be validated (page 211, right column 2nd full paragraph). Only two proteins (agrin and hnBNPm) were then identified individually by Western blot analysis. Zhang et al., refers throughout the publication to these proteins (agrin, hnBNPm) as "protein markers", see, for example page 211, right column, 2nd full paragraph, lines 1-6 and last line; page 214, right column, lines 2-4. Thus, contrary to the Examiner assertion, Zhang et al.,

do refer to these identified proteins as potential markers, but invites further study. Therefore, Zhang et al., actually supports and validates Applicants' study.

Thus, Applicants respectfully submit that the Zhang et al., reference is not applicable to the instant invention and does not control the question of enablement.

Applicants respectfully submit that many of the methods disclosed in the instant specification are routinely practiced in the field of proteomics by those of ordinary skill in the art attempting to identify biomarkers of particular physiological states.

For example, Scott D. Patterson presents the state of the art in mass spectrometry/proteomics by summarizing the Asilomar Conference on Mass Spectrometry (see attached article, Physiological Genomics 2:59-65 2000; reference 1). This conference took place in 2000, thus coinciding with the time that the instant inventors were working to develop the instant invention. For example, at page 64, left column of Patterson is a description of the SELDI approach (as discussed at the conference by Scot Weinberger) wherein defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger described a search

for markers of benign prostate hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These peptides were able to be fragmented using the MALDI-Qq-TOF (a procedure described by Ken Standing at the conference, page 62, left column of Patterson). It was found that there appears to be a difference in the relative level of seminogelin fragments between these two states (prostate cancer and benign prostatic hyperplasia), thus providing a potential differential marker.

Applicants respectfully draw the Examiner's attention to the fact that the method described by Weinberger is analogous to the method described in the instant specification. Furthermore, when interpreting data Weinberger uses the same approach to interpretation as the instant inventors in order to identify seminogelin fragments as a potential marker to distinguish between benign prostate hyperplasia and prostate cancer based on differential expression of the fragments. Additionally, Applicants respectfully point out to the Examiner that Weinberger linked differential expression of seminogelin to benign prostate hyperplasia and prostate cancer without analysis of a sample from a control patient free of disease or analysis of a sample from a

patient having another disease, which is not benign prostate hyperplasia or prostate cancer. Such linking of markers with disease through differential expression is commonly practiced in proteomics.

Considering the above comments, it is clear that both the specification and the prior art disclose how to make and use the instant invention. Accordingly, Applicants respectfully contend that the instant invention satisfies the "test for enablement" since one skilled in the art could make or use the invention from the disclosures in the specification coupled with information known in the prior art without undue experimentation and the specification contains within it a connotation of how to use.

The instant application discloses a method for diagnosing insulin resistance through the detection of the claimed biopolymer marker, SEQ ID NO:2. The data presented in Figures 1, 3 clearly show a positive correlation between the claimed biopolymer marker and insulin resistance. These bipolymer markers have not previously been shown to be associated with insulin resistance. When a marker is discovered to be associated with a disease state, its potential for diagnostics and/or therapeutics is immediately recognized, even if the involvement of the marker in disease pathology is unknown.

As shown by the above arguments, the instant specification

contrary to the Examiner's opinion, does contain proper guidance to enable one of ordinary skill in the art to practice the claimed method for diagnosing insulin resistance without undue experimentation. Thus, the Examiner's argument is not sufficient to support the enablement rejection; since the association of the claimed bipolymer marker, SEQ ID NO:2, with insulin resistance carries with it a connotation of use for diagnostics. Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.

CONCLUSION

In light of the foregoing remarks, amendments to the specification, and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,

Ferris H. Lander
Registration # 43,377

McHale & Slavin, P.A.
2855 PGA Boulevard
Palm Beach Gardens, FL 33410
(561) 625-6575 (Voice)
(561) 625-6572 (Fax)

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news and reports

Mass spectrometry and proteomics

SCOTT D. PATTERSON
Amgen Inc., Thousand Oaks, California 91320-1789

THE 15TH ASILOMAR CONFERENCE on Mass Spectrometry this October was devoted to the role of mass spectrometry (MS) in proteomics. The Asilomar Conference site is in a picturesque national park in Pacific Grove, CA, overlooking the Pacific Ocean. The conference aims to bring together scientists from a cross section of disciplines that are applying MS to an emerging field. This year, that emerging field is proteomics. The term "proteome" was coined by Wilkins et al. (17) in the mid-1990s to describe the protein complement of the genome. The term was first used to describe the 20-yr-old field of two-dimensional gel electrophoresis (2-DE) and quantitative image analysis. 2-DE remains the highest resolution protein separation method available, but the ability to identify the observed proteins was always an extremely difficult problem. MS has been integral to solving that problem. Although improvements in 2-D gel technology had been realized since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics. The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and, more recently, genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and, more recently, the wide-spread introduction of mass spectrometers capable of data-dependent ion selection for fragmentation (MS/MS) (i.e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying the first two technological advances together. Thus MS played a key role in the passage of 2-DE/image analysis to proteomics.

As a note to readers unfamiliar with MS, the instruments are named for their type of ionization source and mass analyzer (see also Refs. 1, 11, 12). To measure the mass of molecules, the test material must be charged (hence ionized) and desolvated (dry). The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer that simply measures the elapsed time from acceleration of the charged (ionized) molecules through a field free drift region. The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC (RP-HPLC) column or a nanospray device (19) that is similar to a microinjection needle. During this process, the droplets containing analyte are dried and gain charge (ionize). The ions formed during this process are directed into the mass analyzer, which could be either a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qq-TOF) type.

This Asilomar meeting provided one of the largest academic forums in the United States for the presentation and discussion of MS as it is applied to proteomics. As is obvious from the introduction, the initial role MS played was as a protein identification and characterization methodology. However, the role of MS is expanding in this field. Although a series of talks focused on the use of different kinds of MS to identify gel-separated proteins and the various automation technologies applied to perform this in high throughput, several talks also presented alternate approaches. These approaches utilized direct analysis of digested protein mixtures for either identification of the components or quantitative analysis of two different samples mixed together. Specific biological applications were also presented. As described above, a critical component of any MS approach as applied to proteomics is the computational analysis. This report will be divided to focus on these six aspects of MS in proteomics. Where references are known for some of the material presented, they are cited. The program was, however, not entirely limited to MS in proteomics. Prior to the six sections covering the conference core, the first section of this report covers those presentations that were aimed at providing an insight into broader biological and drug discovery processes.

Proteomics in biology and drug discovery. The opening lecture, given by Lee Hood (Univ. of Washington), provided an excellent overview of Genomics, Proteomics, and Systems Biology. Hood described the genome project efforts that provide four types of maps: genetic, physical, gene, and sequence. For the human genome,

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it is anticipated that 90–95% of all genes will be sequenced sometime next year. This is the first step toward what Hood described as the "Periodic Table of Life." The different approaches to genomic sequencing and microarray technologies that are able to interrogate the mRNA levels of thousands of genes at a time were described. Hood described proteomics in broad terms as the study of multiplicity of proteins. The information obtained from the various hierarchical levels of biological information (gene, protein, pathways, interconnecting pathways) must be integrated for us to be able to provide a more complete biological picture. For both microarray and proteomics, samples representing the disease process must be obtained. This means that pure cell populations must be microscopically captured from tissues and/or sorted prior to analysis. Therefore, analyses at the mRNA and protein level must be conducted at very low levels and substantial engineering opportunities exist in the biological field to provide the necessary solutions. However, generation of the data is only the first hurdle, as the analysis of the data from a systems perspective then must be undertaken. Hood presented systems biology as the challenge for the 21st century and provided a series of examples of large-scale approaches to biology, from genome sequencing of unicellular organisms, to the sequencing of the T-cell receptor locus, to cancer biology, all of which benefit from such approaches.

Three other presentations were included in the program, to provide a broader background to the utilization of proteomics in drug discovery. Doug Buckley (Exelixis) described the generic view of the drug discovery pipeline, the various "choke" points in the process, and where proteomics could play a role. Of note was the discussion of the changing patent protection landscape, during which Buckley said that full-length cDNA patents were being issued despite the existence of EST patents on portions of these genes. Buckley also predicted that functional data is expected to be required for patents beyond the inferences gained from bioinformatics. The choke points he referred to were target validation, assay development, mechanistic biology, and toxicology. Exelixis is using model organisms (*Caenorhabditis elegans*, *Drosophila*, mouse, and zebrafish) to screen for genes that disrupt/modulate pathways common between man and these organisms. Roles for proteomics included follow-up on targets (direct analysis of protein differences, proteins associated with gene products of interest), assay development [e.g., validation of hits in high-throughput screening (HTS)], and mechanistic biology (e.g. comprehensive analysis of a knockout phenotype). Most importantly, Buckley presented the bottom line that all new technologies must demonstrate their worth by concrete changes in the drug development pipeline (i.e., greater efficiency, better decisions). He predicted that proteomics could provide these benefits at the multiple restriction points referred to above.

Pharmacoproteomics, using 2-DE to profile mechanisms of drug efficacy and toxicity, was presented by Tina Gatlin (Biosource/Large Scale Biology Corporation). The synergy between mRNA expression profiling (for low-abundance gene products) and protein expression profiling (for posttranslational modifications and subcellular localization) was presented. An exception to this is the search for surrogate markers, where secreted proteins were normally the choice and in which there is no identifiable mRNA source to mirror serum or urine protein expression. The aim of their Molecular Effects Database of 2-DE patterns, obtained from livers of drug-treated rats, is to establish links between expression patterns and toxic endpoints to reveal markers for efficacy and prediction of side effects which can be used for lead selection. In disease models, the hypothesis is that the altered expression pattern could be reversed by treatment with a drug.

The closing presentation of the meeting, given by Jeff Seilhamer (Incyte), presented analyses of the precursor to proteins, mRNA. The staff at Incyte have generated very large EST libraries and from these have estimated the number of genes in the human genome to be 129,769 (based on CpG island estimates, 142,634). They are now sequencing the human genome at a rate of about 1 million reads a month on the Megabace platform with 9 sequencing runs/day. Assembly of the data is being accomplished using Linux on 1,500 CPUs (160 computers) with 75 terabytes of storage capacity. Single-nucleotide polymorphisms (SNPs) are being calculated from their sizable EST collection, and mRNA expression profiling is being achieved using their GEM microarray platform. These data are being integrated with 2-DE proteomics data being generated by their partner Oxford GlycoSciences. This integration of the technologies of genomics and proteomics forms the basis of their drug discovery approach for profiling differences between normal and diseased tissue.

Computational aspects of proteomics. Determining the masses of peptides (MS spectra) derived from enzymatic digestion of gel-separated proteins is often the first step in a mass spectrometric-based protein identification strategy. Peptide-mass mapping is the most commonly employed mass spectrometric approach for protein identification from organisms whose genome is completely sequenced (or at least for which the more abundantly expressed genes have been sequenced). The basis of the method is the matching of experimentally determined peptide masses with peptide masses calculated for each entry in a sequence database (using the specificity of the enzyme used to generate the experimental data). How well the experimentally determined masses match with the calculated masses forms the basis of the approach. Ron Beavis (Proteometrics) described how to obtain high-quality data, which even if less, are better than more low-quality data. The use of specific matrices as well as the use of standards with respect to obtaining appropriate data sets for peptide-mass mapping was addressed. Later in the day David Fenyo (Proteometrics) described how to utilize this data in a three-step process as is performed in their WWW-available program, Profound, which uses a Bayesian algorithm (http://www.proteometrics.com). The process is as follows:

1) assignment of monoisotopic masses to the raw data, *2*) peptide-mass search, and *3*) significance testing of the result (4). The last step was presented as the most critical because it is from this that the confidence of the match is derived. This is achieved through calculation of a score frequency function for false positives. This was derived from statistical analysis of the database being searched using random selections of peptide masses from different proteins that are then grouped as synthetic proteins and used in a peptide-mass search of the database in question. This is repeated for a variety of random selections to come up with robust statistics for false positives.

The next level of protein identification is the generation of fragment ion spectra from peptides isolated in the gas phase of the mass spectrometer (MS/MS spectra). Matching of fragment ion spectra follows the same principle as for peptide-mass mapping. Experimentally calculated masses of fragment ions (together with the intact mass of the peptide, and often the specificity of the enzyme used to generate the peptide) are matched with those calculated for isobaric peptides (i.e., same mass as experimentally determined) from entries in sequence databases. Arthur Moseley (Glaxo Wellcome) described how nanoscale capillary LC-MS/MS (where peptides are separated chromatographically before MS/MS) had been automated for identification of gel-separated proteins. The throughput of this 75 μm ID capillary system connected to a Qq-TOF mass spectrometer was 20 samples per day at levels to 30 fmol (loaded on gel) for BSA. Moseley continues to develop ultra-HPLC (in some cases combined with variable flow systems) that improve both the speed and resolution of separation. In a Glaxo Organelle Proteomics program, various approaches to protein identification were examined. A comparison of the total number of proteins identified following in situ enzymatic digestion of proteins separated by either high-resolution 2-DE or one-dimensional (usually SDS-PAGE) gel electrophoresis (1-DE) was presented. Only one or a limited number of proteins are present in each of the 2-DE spots, whereas many proteins were present in the 1-DE bands of the enriched Golgi complex. In fact, more proteins were identified from the 1-DE bands than from the 2-DE spots (see below, *Analysis of complex protein mixtures without gel electrophoresis*).

MS/MS spectra derived from tryptic digestions conducted in the presence of equal quantities of $H_2{}^{16}O$ and $H_2{}^{18}O$, when combined with subtractive analysis of the two types of spectra, allows de novo sequencing as described by Matthias Wilm (EMBL) (18). By utilizing a Qq-TOF mass spectrometer, peptides containing both COOH-terminally incorporated stable isotopes and just the isoform containing the ^{18}O could be selected for fragmentation from the mixture. Subtraction of the ^{18}O spectrum from the ^{16}O:^{18}O spectrum reveals only ^{16}O *y*-series ions. Often, a complete ion series is obtained. The method has proved feasible in their hands when 1 pmol of protein is present in the gel (1/4 of this amount can be successfully analyzed with standard digest conditions).

Automated identification of gel-separated proteins by mass spectrometry. Following quantitative analysis of 2-DE patterns, the next step is the identification of all protein spots that display differential expression. Andrew Gooley (Proteome Systems) described the approaches they are employing for quantitative analysis using 2-DE. This included the following: sample preparation (sequential detergent extraction with aminosulfobetaine-14), narrow-range immobilized pH gradient (IPG) with mini-gels for the 2nd dimension, through to the robotic system that they have codeveloped for spot excision, liquid handling (peptide extraction and reverse-phase bead cleanup and storage) and peptide-mass fingerprinting by MALDI-MS. Apart from the throughput of the robotic system, diminished contamination from keratin and more reproducible spotting of samples for MALDI-MS is a highly desirable feature of automation. Hans-Werner Lahm (Hoffmann-La Roche) described the high-throughput system they use for automated spot excision from 2-DE, digestion (with low-salt buffer to eliminate the need for cleanup), and spotting for automated MALDI-MS. Lahm also described the computational aspects of operating such a system in high-throughput mode for long periods of time, including automated database search routines for users distributed throughout the world at other Roche sites. They are investigating the use of stable isotope labeling ($^{14}N/^{15}N$) followed by mixing of each sample prior to 2-DE for direct quantitation of relative expression differences from the MALDI-MS spectra of individual protein spots. The system averages 1,000 spots to spectra per day (including downtime).

David Arnott (Genentech) described automation of in-gel digestions following analysis of differentially regulated proteins from 2-DE. Arnott described the trapping cartridge approach that was required to analyze extracted peptides from the DigestPro robot (currently 30 sample spots, but upgradeable to 96) by microcapillary LC-MS/MS. They aimed to automate as much of the sample processing as possible with automated liquid handling from the digestion robot to the data-dependent LC MS/MS (capable of handling 40 samples per day) using an ion-trap mass spectrometer followed by auto-database searching using Sequest (3). The system is capable of analysis of subpicomolar quantities of protein from silver-stained gels.

Advances in separations and mass spectrometers. Accurate mass analysis of intact proteins using an 11.5-T FT-ICR coupled with a capillary electrophoresis (CE) instrument was demonstrated by Richard Smith (Pacific Northwest National Laboratory) as a means of proteome analysis. Through the use of stable isotope labeling of one sample and running that sample with an unlabeled sample provides the possibility to measure protein expression ratios. To identify the proteins that display different ratios, dissociation in the FT-ICR-MS to yield mass tags is possible. Having intact mass information as well as identification allows post-translational modifications to begin to be investigated. The mass accuracy obtainable by this FT-ICR-MS was said to be <0.75 ppm which allows the generation of

accurate mass tags for tryptic peptides. In many cases this may be sufficient for protein identification (at least for an organism like *C. elegans*). In some cases, MS/MS may be required, but once performed it would not have to be repeated. Another possibility is the identification of cysteine-containing peptides at a mass accuracy of 1 ppm, which was said to be sufficient for identification. Another possibility being explored with this instrument is multiplexed MS/MS, where up to 7 ions could be isolated at once and the MS/MS spectrum could be deconvoluted for each selected ion (requires accuracy of <10 ppm). This will be tried with online separations in the near future.

Marvin Vestal (PE BioSystems) described his continuing efforts in MALDI-MS instrument design. The attributes he is aiming for include sensitivity, specificity (resolution, mass accuracy, selective ionization), speed, accuracy, dynamic range, and mass range. The sensitivity will always be limited by chemical noise, but the aim is to reduce the limitations of ionization and data handling. Vestal would like to achieve a sensitivity of 1 fmol with a data acquisition rate of 1 spectrum/s. The instrument he is designing to achieve these aims is a MALDI-TOF/TOF-MS. This system has an ion gate (with 500 resolution and no loss of sensitivity) after the collision cell so that metastable ions created after reacceleration are removed. Although this system is in the early stages of development, the data shown demonstrate that this instrument is meeting most of the stated objectives.

The hybrid quadrupole TOF (Qq-TOF) mass spectrometer developed a few years ago (9), which has now been commercialized, utilizes an ESI for ionization (10). Both Ken Standing (Univ. of Manitoba) and Brian Chait (Rockefeller Univ.) described the use of a MALDI ion source for introduction of ions into a modified commercial Qq-TOF, thus taking advantage of both the high efficiency ion production of the MALDI and the ion isolation/fragmentation of the quadrupole system with a TOF mass analyzer. Standing presented data showing sensitivity of purified standards (e.g., Substance P) in the 70 amol range (1-min acquisition) for MS and 7 fmol for MS/MS with 10,000 resolution. This instrument offers similar advantages to the TOF/TOF described above.

Online MS analysis of capillary electrophoretic or chromatographic separations of peptide (or proteins) is most often achieved using ESI-MS. Barry Karger (Barnett Institute) described how very small quantities of peptides/proteins could be separated and analyzed using vacuum deposition onto Mylar audio tape for subsequent coupled MALDI-MS analysis. The approach had so far been multiplexed with the effluent of 12 capillaries being deposited under vacuum onto the tape. The approach is designed for high-throughput separations and mass analysis.

Proteomic analyses often employ 2-DE, but David Lubman (Univ. of Michigan) described a liquid-phase 2-D separation of proteins utilizing a novel MS. The requirements of his mass spectrometer were high sensitivity, low duty cycle, and fast response. He designed and built an ion trap to capture ions from the CE coupled to TOF-MS. The 2-D liquid phase separation consisted of nonporous silica bead RP-HPLC (which provided good resolution <50 kDa) that was conducted at high pH followed by CE and MS. Whole cell lysates were analyzed with this system, and some of the data obtained were presented.

Biological applications. Brian Chait (Rockefeller Univ.) presented the culmination of an enormous amount of work at both the protein chemistry (mass spectrometry) and cell biology levels. The nuclear pore complex (NPC) in yeast is a massive structure (1,000 Å across with 8-fold symmetry) that regulates protein transport in/out of the nucleus. The first step in understanding this structure was to purify the complex and then identify every protein present. The protein fraction was separated by several different chromatographic steps followed by SDS-PAGE from which every visible band was excised and analyzed by MALDI-IT-MS. This was an especially daunting task as the NPC when isolated contains a snapshot of the proteins transiting the NPC at that point in time. Hence, of the 174 proteins identified, 29 were nucleoporins and only 14 were shown to be present in the NPC. These 14 proteins were characterized as being present in the NPC by a variety of techniques. Protein A (4.5 repeats of the Fc binding region) fusions with the proteins of interest were generated, and immunohistochemistry was performed on cells transfected with these constructs. Electron microscopy of hundreds of NPCs following transfection allowed stoichiometry and symmetry (nuclear/cytoplasmic/asymmetric) to be determined. Subcellular fractionation and high-pH extractions were also performed to further characterize localization biochemically. This elegant study has allowed a testable model for nuclear transport to be constructed.

Two examples of the utility of analysis of unfractionated or partially fractionated complex protein mixture digests (see next section) were presented by Scott Patterson (Amgen Inc.). As a first step in the understanding of the interchromatin granule clusters (IGC), a nuclear organelle which is a major site of mRNA splicing. Samples enriched in this structure were digested with trypsin, and the complex mixture of peptides was analyzed by data-dependent LC-MS/MS (8). Some proteins known to be present in these structures were identified together with 19 novel genes (including ESTs). Three of the genes were confirmed to be present in the IGC by immunohistochemistry of cells transfected with yellow fluorescent protein (YFP)-fusion constructs with counter staining of splicing factors. The other study presented identified 108 proteins present in a protein fraction obtained from isolated mitochondria treated with atractyloside [mimicking in vitro the permeability transition pore complex (PTPC) which occurs during apoptosis] (13).

Analysis of immunoprecipitates using a new affinity strategy was presented by Gitte Neubauer (EMBL). The new strategy is referred to as tandem affinity purification (TAP) and was developed by colleagues at EMBL (15). The system utilizes a double tag for higher

specificity and much reduced background. The human spliceosome immunoprecipitated under normal conditions (see Ref. 5 for same approach with yeast tri-snRNP) and using the TAP method were compared, demonstrating the utility of this approach.

The common theme of all of these applications is that MS was utilized early on to provide rapid and accurate protein identifications. The genes identified could then be further analyzed to attempt to determine their function.

The use of MS to identify proteins from 2-DE gels was also described by Al Burlingame (Univ. of California, San Francisco) and Reid Townsend (Oxford GlycoSciences). Burlingame described their work to identify protein targets of acetaminophen during acute toxicity and the intricacies of such analyses (14). Townsend described an Oxford GlycoSciences and Pfizer collaboration to separate by 2-DE and identify proteins from cerebrospinal fluid (CSF) in a study aimed at identifying markers for Alzheimer's disease. CSF is a compartment isolated by the blood-brain barrier but it is not just a filtrate of blood. It is produced by the choroid plexus and has a total central nervous system volume of about 90–150 ml that is turned over a few times per day. Comparative analysis of matched plasma CSF samples (in addition the normal/diseased samples) revealed that key plasma proteins (e.g., albumin, transferrin, IgG) showed markedly different relative ratios between plasma and CSF. For effective 2-DE analysis of these samples, a selective removal of albumin, IgG, transferrin, and haptoglobin had to be developed. This was accomplished by affinity depletion. Interestingly, many features in a 2-DE separation are albumin fragments (in fact, 4% of total features). Their study included 512 samples from 228 patients and resulted in 1,131 features (spots) being annotated. Potential markers of Alzheimer's disease were said to be identified.

Separate from the MS identification issues covered in most of the meeting, Kerstin Strupat (Univ. of Muenster) presented her work on MS analysis of noncovalent complexes. Here the challenge is to transfer noncovalent interactions that occur in the condensed phase to the gas phase. ESI-MS has been shown by a number of groups to work, but MALDI-MS analysis has proved more difficult. Examples of MALDI-MS analysis of noncovalent protein:protein (streptavidin tetramer and the macrophage migration inhibitory factor related proteins MRP-8 and MRP-14) and protein:ligand (aldose reductase:NADP) interactions were presented. Interestingly, analysis of the first laser pulse during a MALDI-MS analysis often allows investigation of noncovalent interactions that are not observed during subsequent pulses (16).

Analysis of complex protein mixtures without gel electrophoresis. The first stage of many proteome projects is the identification of the components comprising the system under study. This is of course the first step in understanding any biological system. As described above, an increasing (but still limited) number of laboratories have access to robotic systems requisite for the analysis of large numbers of spots from 2-DE. However, a trend in the field is emerging toward the elimination of the high-resolution protein separation step prior to protein identification by MS. In this approach, the entire enriched protein fraction is enzymatically digested (usually with trypsin), and the resulting complex peptide mixture is subjected to data-dependent LC-MS/MS. In this approach the peptides are separated by both hydrophobicity (RP-HPLC) and charge (m/z in the mass spectrometer) prior to ion selection by the MS control software (hence, data dependent). At this meeting, presentations from five groups demonstrated the utility of the approach to identify components of complex mixtures.

Analysis of immunoprecipitated proteins or enriched protein fractions (e.g., Golgi complex) by either gel electrophoresis followed by in-gel digestion and MS or digestion of the entire protein fraction and analysis by data-dependent LC-MS/MS using a Qq-TOF was described by Jyoyti Choudhary (Glaxo Wellcome). Batched MS/MS spectra were searched using the Mascot program (**http://www.matrixscience.com**). Data presented showed that if the immunoprecipitate was clean, then direct digestion of the mixture proved slightly more successful than analysis of gel-separated proteins. When an enriched Golgi complex from rat liver was separated by either 2-DE (135 spots) or 1-DE (77 bands) and in-gel digested followed by LC-MS/MS, significantly more proteins were identified from the 1-DE separation.

David Arnott (Genentech) described the proteomics component of Genentech's Secreted Protein Discovery Initiative, which also includes genomic, signal trap, expression, and functional analysis. Arnott evaluated three methods to identify proteins secreted from human umbilical microvascular endothelial cells (HUMECs) into 60 ml of serum-free media; 2-DE and 1-DE (with/without staining) followed by in-gel digestion, and direct digestion of the entire protein mixture. Digests were analyzed using the microcapillary system described above. Interestingly, direct digestion followed by data-dependent LC-MS/MS identified the most proteins, but all three methods were complementary in their hands (21 proteins identified by all three methods but no completely novel gene products).

Analysis of serum fractionated using the Cohn pH/ethanol precipitation protocol followed by digestion of the entire fraction prior to data dependent LC-MS/MS was described by Karl Clauser (Millennium Pharmaceuticals) in the context of the studies of differences between wild type and ApoE −/− mice. Clauser also presented the bioinformatics flow for data handling, which utilizes a variant of the publicly available MS-Tag (**http://prospector.ucsf.edu/**) for protein identification and a de novo sequence interpretation program referred to as SHERENGA (2). Their stated aim is for searching to keep up with the LC-MS/MS. They have also been experimenting with the JEX ion-exchange protocol developed by Andy Link (7) as a means of decreasing the complexity of the sample and reducing the number of singly charged and highly charged ions as these are less likely to be identified. In one IEX

fraction, 87 plasma proteins were identified in a single run compared with 66 from an unfractionated sample.

Scott Patterson (Amgen) described Amgen's proteomics efforts, now in the third year. They are employing data-dependent LC-MS/MS of complex protein mixture digests. The stated aim is to reduce the complexity such that in an ideal situation only one peptide for each protein in the mixture is fragmented during LC-MS/MS. To achieve this aim, various affinity methods can be employed, and the use of cysteinyl peptide capture using either thiopropyl Sepharose or a biotin alkylating reagent, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (HPDP-biotin), was described (13). The former was used in a large-scale analysis of urinary proteins where digestions of the unfractionated starting material, albumin/IgG depleted, or cysteinyl peptide captured or noncaptured were analyzed. The samples were analyzed with replicate LC-MS/MS runs using narrow mass ranges for ion selection for each run, thereby increasing the number of unique spectra selected for fragmentation. This analysis resulted in the identification of over 200 proteins, including a number of uncharacterized nucleotide sequences (e.g., ESTs). Smaller scale analyses are described above, in one case [soluble intermembrane proteins (SIMPs)] utilizing cysteinyl peptide capture to identify more proteins than with no fractionation. Data handling for this high-throughput effort was also briefly described. A number of the fractions being analyzed have some of the same components; therefore, to enhance the identification process, spectral matching of the database (>5 million spectra) is performed. This links identical spectra and therefore reduces the redundancy associated with re-searching already identified spectra.

Quantitative analysis of two samples without electrophoresis. MALDI-MS, using the surface enhanced laser desorption ionization (SELDI) surface, to search for disease markers in biological fluids was presented by Scot Weinberger (Ciphergen Biosystems). In this approach, defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger described the search for markers of benign prostatic hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These were able to be fragmented using the MALDI Qq-TOF of Standing, described above. It appears as though there is a difference in the relative level of a seminogelin fragment between these two diseases, providing a potential differential marker. The method is sensitive but apparently limited to analysis of proteins less than about 20 kDa (a range not well characterized by 2-DE).

A combination gel/MS approach referred to as a "virtual 2-D gel" was presented by Phil Andrews (Univ. of Michigan). In this approach, proteins are separated by charge using thin-layer isoelectric focusing (IEF), and this gel is then subjected to MALDI-MS. By rastering through the entire IEF gel, a composite display of all acquired MALDI-MS spectra can be generated (hence, the virtual 2-DE). Such analyses would provide very accurate mass measurements, greatly assisting in posttranslational modification analyses as well as potentially quantitation.

Karl Clauser (Millennium Pharmaceuticals) described their efforts at utilizing already existing LC-MS/MS data to attempt to gain some quantitative/qualitative information as to differences between samples. Differences in serum protein levels between wild-type and ApoE −/− mice have been examined using this approach, which compares the MS ion current from peptides identified between LC-MS/MS runs of each sample. Comparison between runs is a difficult task, but data suggested that there is sufficient confidence to state a significant difference if there is a difference of a factor of 3 between some components of the samples.

An LC-MS/MS-based system was described by Steve Gygi (Univ. of Washington) for quantitative analysis of complex mixtures. The technology is referred to as isotope-coded affinity tag (ICAT) (6). The ICAT reagent described here is composed of three units: an affinity reagent (biotin), a linker region (one of two forms), and a reactive group (a thiol-specific reagent, iodoacetic acid). The linker region encodes the mass difference, with the light version having 8 hydrogens and the heavy version having 8 deuteriums. Thus the mass difference is 8 mass units (doubly charged ions will have an m/z difference of 4). Following reduction and alkylation of each of the two protein samples with one of the two reagents, the two samples can then be mixed together. All subsequent manipulations are performed as a mixture, culminating in tryptic digestion of the complex sample and capture of the cysteinyl peptides on avidin. The bound peptides are released and analyzed by LC-MS/MS, revealing paired signals of peptides. Calculation of areas under the peak for each paired ion from the LC-MS data provides an accurate record of the relative quantities of the proteins from each starting sample. The MS/MS spectra allow identification of the peptides. The approach was elegantly demonstrated with yeast grown on either galactose-containing media or ethanol-containing media. Proteins expected to be differentially regulated were observed, and, highlighting the advantages of analysis at the protein level as opposed to the mRNA level (e.g., microarray), alcohol dehydrogenase-1 (ADH1) was found to be oppositely regulated (as expected) to ADH2, to which it is 95% homologous. This is a very promising approach for quantitative analysis of complex protein mixtures.

A number of interesting posters were also presented at the meeting, and some of the presenters were given the opportunity to "advertise" their posters. These dealt with the same range of subjects presented in the oral sessions.

Conclusion. The organizers Ruedi Aebersold and John Stults brought together an excellent program for this meeting, with essentially all major laboratories in

this field being represented. The field has grown enormously over the past few years, and advancements presented at this meeting indicate an optimistic view of the future for proteomics. This very successful meeting provided the 162 attendees with the state-of-the-art in mass spectrometry and proteomics.

Address for reprint requests and other correspondence: S. D. Patterson, Biochemistry, Amgen Inc., One Amgen Center Drive, MS 14 2 E, Thousand Oaks, CA 91320-1789 (E-mail: spatters@amgen.com)

REFERENCES

1. **Carr SA and Annan RS.** Overview of peptide and protein analysis by mass spectrometry. In: *Current Protocols in Molecular Biology*, edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. New York: Wiley, 1997, p. 10.21.1–10.21.27.
2. **Dancik V, Addona TA, Clauser KR, Vath JE, and Pevzner PA.** De novo peptide sequencing via tandem mass spectrometry. *J Comput Biol* 6: 327–342, 1999.
3. **Eng JK, McCormack AL, and Yates JR, III.** An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5: 976–989, 1994.
4. **Eriksson J, Chait BT, and Fenyo D.** A statistical basis for testing the significance of mass spectrometric protein identification results. *Anal Chem* In press.
5. **Gottschalk A, Neubauer G, Banroques J, Mann M, Luhrmann R, and Fabrizio P.** Identification by mass spectrometry and functional analysis of novel proteins of the yeast [U4/U6.U5] tri-snRNP. *EMBO J* 18: 4535–4548, 1999.
6. **Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R.** Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999, 1999.
7. **Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, and Yates JR, III.** Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol* 17: 676–682, 1999.
8. **Mintz PJ, Patterson SD, Neuwald AF, Spahr CS, and Spector DL.** Purification and biochemical characterization of interchromatin granule clusters. *EMBO J* 18: 4308–4320, 1999.
9. **Morris HR, Paxton T, Langhorne J, Berg M, Bordoli RS, Hoyes J, and Bateman RH.** High sensitivity collisionally-activated decomposition tandem mass flight mass spectrometer. *Rapid Commun Mass Spectrom* 10: 889–896, 1996.
10. **Morris HR, Paxton T, Panico M, McDowell R, and Dell-A.** A novel geometry mass spectrometer, the Q-TOF, for low femtomole/attomole-range biopolymer sequencing. *J Protein Chem* 16: 469–479, 1997.
11. **Patterson SD.** Protein identification and characterization by mass spectrometry. In: *Current Protocols in Molecular Biology*, edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. New York: Wiley, 1998, p. 10.22.1–10.22.24.
12. **Patterson SD and Aebersold R.** Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* 16: 1791–1814, 1995.
13. **Patterson SD, Spahr CS, Daugas E, Susin SA, Irinopoulou T, Koehler C, and Kroemer G.** Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition. *Cell Death Differ* In press.
14. **Qiu YC, Benet LZ, and Burlingame AL.** Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem* 273: 17940–17953, 1998.
15. **Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, and Seraphin B.** A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17: 1030–1032, 1999.
16. **Vogl T, Roth J, Sorg C, Hillenkamp F, and Strupat K.** Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 10: 1124–1130, 1999.
17. **Wilkins MR, Sanchez J C, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, and Williams KL.** Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* 13: 19–50, 1995.
18. **Wilm M, Neubauer G, and Bachi A.** Automatic de novo sequencing of proteins by differential scanning with a quadrupole time of flight instrument. *Proc 47th ASMS Conf Mass Spectrom Allied Topics Dallas June 13–17 1999*, p. 1541.
19. **Wilm MS and Mann M.** Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *Int J Mass Spectrom Ion Processes* 136: 167–180, 1994.

Mass spectrometry and proteomics

SCOTT D. PATTERSON

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